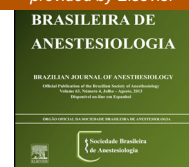




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SCIENTIFIC ARTICLE

Evaluation of genotoxicity induced by repetitive administration of local anaesthetics: an experimental study in rats

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Abstract

Background and objective: Previous studies regarding the effects of some local anaesthetics have suggested that these agents can cause genetic damage. However, they have not been tested for genotoxicity related to repetitive administration. The aim of this study was to evaluate the genotoxic potential of local anaesthetics upon repetitive administration.

Methods: 80 male Wistar rats were divided into: group A – 16 rats intraperitoneally injected with lidocaine hydrochloride 2%; group B – 16 rats IP injected with mepivacaine 2%; group C – 16 rats intraperitoneally injected with articaine 4%; group D – 16 rats IP injected with prilocaine 3% (6.0 mg/kg); group E – 8 rats subcutaneously injected with a single dose of cyclophosphamide; and group F – 8 rats intraperitoneally injected with saline. Eight rats from groups A to D received a single dose of anaesthetic on Day 1 of the experiment; the remaining rats were dosed once a day for 5 days.

Results: The median number of micronuclei in the local anaesthetics groups exposed for 1 or 5 days ranged from 0.00 to 1.00, in the cyclophosphamide-exposed group was 10.00, and the negative control group for 1 and 5 days was 1.00 and 0.00, respectively ($p < 0.0001$). A significant difference in the number of micronuclei was observed between the cyclophosphamide group and all local anaesthetic groups ($p = 0.0001$), but not between the negative control group and the local anaesthetic groups ($p > 0.05$).

Conclusion: No genotoxicity effect was observed upon repetitive exposure to any of the local anaesthetics evaluated.

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PALAVRAS-CHAVE

Anestesia;
Testes de
mutagenicidade;
Testes para
micronúcleos;
Prilocaína

Avaliação da genotoxicidade induzida pela administração repetida de anestésicos locais: um estudo experimental em ratos

Resumo

Justificativa e objetivos: Estudos anteriores sobre os efeitos de alguns anestésicos locais sugeriram que esses agentes podem causar alterações genéticas. No entanto, esses agentes não são testados para genotoxicidade relacionada à administração repetida. O objetivo deste estudo foi avaliar o potencial genotóxico de anestésicos locais após repetidas administrações.

Métodos: 80 ratos Wistar machos foram alocados em: grupo A – 16 ratos receberam injeção por via intraperitoneal (IP) de cloridrato de lidocaína a 2%; grupo B – 16 ratos receberam injeção IP com mepivacaína a 2%; grupo C – 16 ratos receberam injeção IP de articaína a 4%; grupo D – 16 ratos receberam injeção IP de prilocaína a 3% (6,0 mg kg⁻¹); grupo E – 8 ratos receberam injeção subcutânea em dose única de ciclofosfamida; grupo F – 8 ratos receberam injeção IP com solução salina. Oito ratos dos grupos de A a D receberam uma dose única de anestésico no Dia 1 da experiência; os ratos restantes foram dosados uma vez por dia durante cinco dias.

Resultados: A mediana do número de micronúcleos nos grupos com anestésicos locais expostos por um ou cinco dias variou de 0,00 a 1,00; no grupo exposto à ciclofosfamida foi de 10,00 e no grupo controle negativo no primeiro e quinto dias foi de 1,00 e 0,00, respectivamente ($p < 0,0001$). Uma diferença significativa foi observada no número de micronúcleos entre o grupo ciclofosfamida e todos os grupos com anestésicos locais ($p = 0,0001$), mas não entre o grupo controle negativo e os grupos com anestésicos locais ($p > 0,05$).

Conclusão: Nenhum efeito de genotoxicidade foi observado após a exposição repetida a qualquer um dos anestésicos locais avaliados.

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Introduction

The development of safe and effective local anaesthetic agents has been one of the most important advances in dental science over the last century. The dental agents currently available are extremely safe and meet most of the criteria for an ideal local anaesthetic. These local anaesthetic agents induce minimal tissue irritation and have a low risk of inducing allergic reactions.¹

A local anaesthetic is most often used in dentistry to control pain and is also widely used in other fields of medicine. Among the various formulations of local anaesthetics, the most commonly used types are anaesthetic salts of lidocaine, mepivacaine and prilocaïne.²

The combined use of a vasoconstrictor and a local anaesthetic agent was first reported in 1901, when Braun simultaneously administered adrenaline and cocaine.³ Due to the vasodilation properties of most anaesthetic salts, the duration of anaesthesia is not always suitable, illustrating the necessity of concomitant administration with a vasoconstrictor. Some advantages of the combined administration of vasoconstrictors and anaesthetics are the slow absorption of the anaesthetic salt (which reduces toxicity and increases the duration of anaesthesia), a reduction in the quantity of anaesthetic required to anaesthetise the patient and an increase in the effectiveness of the anaesthetic.² The most common vasoconstrictors used in combination with local anaesthetics belong to the group of sympathomimetic amines, which includes adrenaline, noradrenaline, levonordefrin, phenylephrine and felypressin.²

Genotoxic agents negatively affect the integrity of a cell's genetic material and are defined as any substance

or chemical that damages DNA. Although the ability of a substance to damage DNA does not automatically render it as a health hazard, it does raise concerns that the substance may be a potential mutagen and/or carcinogen.⁴

Some local anaesthetics have not been tested for carcinogenicity or genotoxicity. Prilocaïne is a local anaesthetic that has been under review by the National Toxicology Program (NTP, USA) since October 2007.⁵

The micronucleus test is widely used to evaluate the ability of a substance to break chromosomes (referred to as its clastogenicity) or affect the formation of the mitotic metaphase plate and/or spindle, both of which can lead to inequitable distribution of chromosomes during cellular division.⁶ The micronucleus test generates results with strong statistical support; therefore, it is widely used as a screening tool to determine the safety of many substances and to classify agents as carcinogenic or non-carcinogenic.⁷ The ease of implementation of the micronucleus test has led to widespread adoption worldwide as a standard genotoxicity test to monitor the safety of agents for use in the human population.⁸

To the best of our knowledge, there are no studies in the literature addressing the genotoxic potential of the repetitive use of local anaesthetics. Local anaesthetics are widely used in dentistry and medicine, and studies that evaluate the risk of repetitive exposure to these substances may contribute to a better understanding of their potentially toxic effects on genetic material and their potential risk to exposed patients.

The objective of this study is to investigate the genotoxic potential of the repetitive use of local anaesthetics using the micronucleus test.

Methods

This study was approved by the Ethics Committee on Animal Use (Protocol n° 930/11).

For this study, 80 male Wistar rats weighing between 200 and 250 g were administered local anaesthetics at 12 weeks of age. The rats were divided into groups of four in large rectangular boxes (measuring 49 cm × 34 cm × 16 cm) suitable for the accommodation of up to five adult rats. The rats were placed in a temperature- and humidity-controlled vivarium equipped with a 12-h light/dark cycle light. All animals were weighed prior to anaesthetic administration to calculate the proper dosage.

The animals were divided into 6 groups: group A – 16 rats intraperitoneally (IP) injected with lidocaine hydrochloride and phenylephrine (Novocol® 100, SS White, Rio de Janeiro, Brazil) at a dose of 4.4 mg/kg, group B – 16 rats IP injected with mepivacaine 2% (mepivacaine, DFL, Jacarepaguá, Brazil) at a dose of 4.4 mg/kg, group C – 16 rats IP injected with epinephrine and articaine 4% (Septanest® 1:100,000, Septodont, Brussels, Belgium) at a dose of 7.0 mg/kg, group D – 16 rats IP injected with prilocaine 3% and felypressin (Cytanest®, Astra, São Paulo, Brazil) at a dose of 6.0 mg/kg, group E – 8 rats subcutaneously injected with a single dose of cyclophosphamide (Genuxal®, Baxter Oncology GmbH, Halle/Westfalen, Germany) (50 mg/kg) on Day 1 of the experiment (positive control group),⁸ and group F – eight mice IP injected with 0.5 mL of saline (negative control group). Because a previous report demonstrated the formation of micronuclei in response to cyclophosphamide at a dose of 50 mg/kg, this dose was used for the positive control group.⁸

Eight rats from groups A to D received one dose of anaesthetic on Day 1 of the experiment. The remaining animals in these groups received a daily dose of anaesthetic for five days. The rats in group F were administered saline in a similar manner.

Eight rats from groups A to D, four rats from group F, and all of the rats in group E were euthanised 24 h after administration of the anaesthetic. The remaining animals of groups A, B, C, D and F were euthanised 5 days later. Euthanasia was performed with sodium pentobarbital (Syntec, Cotia, São Paulo, Brazil) at a dose of 100 mg/kg by IP injection.

Bone marrow samples were collected from the femur of each rat at the time of sacrifice, and two sample slides were prepared per animal.⁸ The slides were stained with Giemsa stain (Dolles, São Paulo, Brazil). Two thousand polychromatic erythrocytes (1000 per slide) were counted for each animal at a magnification of 400× using an optical microscope to determine the number of micronuclei.⁸ Micronuclei were defined as structures with probable halos surrounding the nuclear membrane and a volume of less than one-third that of the diameter of the associated nuclei; the micronuclei staining intensity was similar to the intensity of the associated nuclei, and both structures were observed in the same focal plane.⁹ The slide analysis was performed in a blinded manner by a single person (MCO) and reviewed by a second person (GAN); both results were concordant.

Statistical analysis

The variance in micronuclei frequency did not have a normal distribution when analysed by the Kolmogorov–Smirnov test ($p=0.0001$) nor were the variances homogenous ($p=0.004$) by Levene test analysis. We therefore chose to use the nonparametric Kruskal–Wallis test followed by multiple comparisons with the Student–Newman–Keuls test to determine statistical significance. All statistical tests were performed at a significance level of 5%.

Results

The median number of micronuclei observed for each group was as follows: for the lidocaine group, the 1- and 5-day exposures were 1.00 and 0.50, respectively; for the mepivacaine group, the 1- and 5-day exposures were both 1.00; for the articaine group, the 1- and 5-day exposures were 1.00 and 0.00, respectively; and for the prilocaine group, the 1- and 5-day exposures were both 0.00. In the group exposed to cyclophosphamide (the positive control), the median number of micronuclei was 10.00. The negative control groups for days 1 and 5 had median numbers of micronuclei of 1.00 and 0.00, respectively ($p<0.0001$) (Figs. 1 and 2 and Table 1).

The number of micronuclei in the positive control group (cyclophosphamide) significantly differed from those observed for all of the local anaesthetics studied, both in the type of anaesthetic administered and the time of exposure ($p=0.0001$). However, no significant difference in the number of micronuclei was observed between the negative control group and the local anaesthetic groups, regardless of the type of anaesthetic administered or the time of exposure ($p>0.05$) (Table 1).

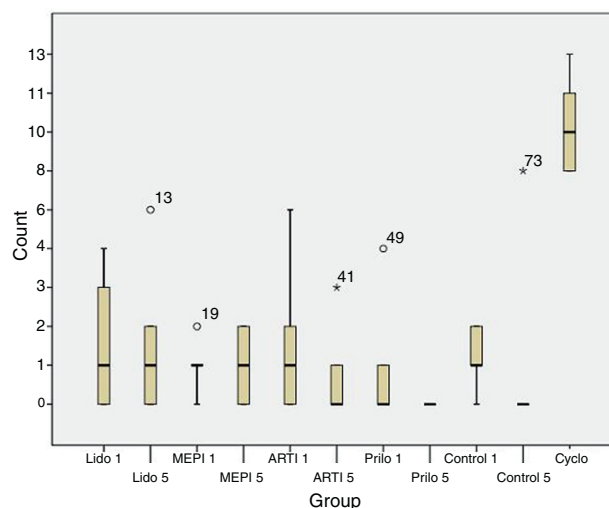


Figure 1 Micronuclei counts per study group (median and interquartile intervals). LIDO, lidocaine; MEPI, mepivacaine; ARTI, articaine; PRILO, prilocaine; CONTROL, negative control; CYCLE, cyclophosphamide (positive control); 1, exposure for 1 day; 5, exposure for 5 days; ○, outlier; *, the outlier of the outlier; the numbering over the outlier corresponds to the numbering of animals.

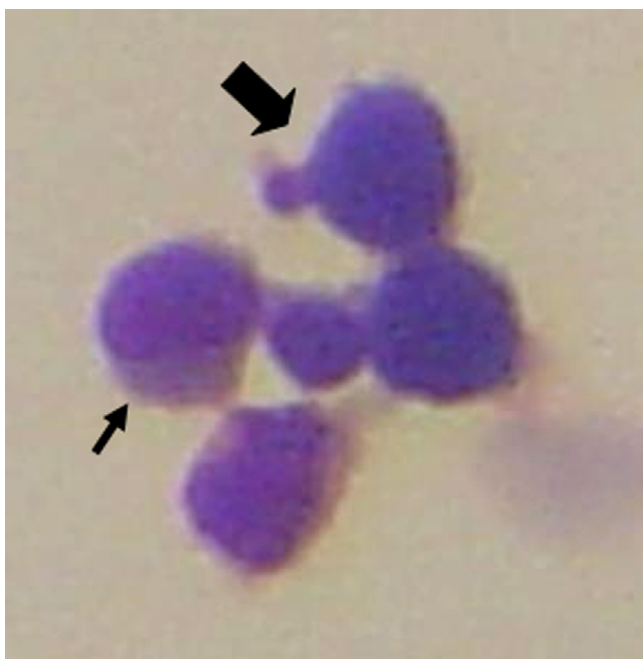


Figure 2 Examples of a polychromatic erythrocyte with a micronucleus (large arrow) and a normal polychromatic erythrocyte (narrow arrow); the animal was exposed to mepivacaine for 5 days (Giemsa staining, 1000 \times).

With the exception of the cyclophosphamide group, all groups were equal for multiple statistical analyses. However, significant differences in the frequencies of micronuclei were observed between the prilocaine 5-day exposure group and the following groups: lidocaine 1-day exposure group ($p=0.0466$), the mepivacaine 1- ($p=0.0437$) and 5-day ($p=0.0460$) exposure groups and the articaine 1-day exposure group ($p=0.0364$) (Table 1).

Discussion

Genotoxicity tests are important for the evaluation of cellular toxicity and the identification of potential carcinogens and mutagens. Several techniques are employed to test

an agent's genotoxic activity, including assays that determine DNA/protein cross-linking coefficients, mitochondrial enzymatic activity, cell proliferation, repair of DNA breaks, mitotic index, the type of damage incurred, chromosomal aberrations, chromosomal non-disjunctions, and levels of apoptosis and necrosis.⁸ The micronucleus test has been used extensively to test the genotoxicity of many chemicals. Micronuclei are easily viewed in erythrocyte samples and are strongly indicative of chromosomal aberrations.⁶

The micronucleus test was first reported in 1970 by Boller and Schmid and was subsequently used by Heddle in 1977.¹⁰ The micronucleus is an additional nucleus, separated from the main nuclear core of a cell during cell division and comprises whole chromosomes or chromosomal fragments that lag behind the other chromosomes upon the completion of mitosis. The micronucleus results from spontaneous or experimentally induced structural changes in the chromosome(s), or through cellular fusion errors, and it is therefore excluded from the new nucleus that is re-formed in telophase.¹¹

The advantages of the micronucleus assay over other tests used to diagnose diseases and monitor environmental contaminants include its simplistic analysis, its high detection sensitivity and accuracy of chromosome losses and nondisjunction events, its ability to measure the length and progression of nuclear division and its ability to detect repair and excision events.⁸ These advantages prompted us to choose the micronucleus test to evaluate the genotoxic effects of repetitive administration of local anaesthetics in this study.

A disadvantage of using vasoconstrictors in conjunction with local anaesthetics is apparent with intravascular injections, during which high concentrations and large volumes can lead to intoxication.¹² Thus, in some patients, it is unacceptable to use vasoconstrictors, particularly in those with diabetes or heart disease or pregnant women. In these cases, the most commonly used anaesthetic salt in the absence of a vasoconstrictor is mepivacaine.¹² Thus, mepivacaine was used in this study without a vasoconstrictor. There are no known reports in the literature that evaluate the genotoxic action of vasoconstrictors.

Prilocaine was previously reported to exhibit genotoxic activity in somatic cells and to be capable of inducing homologous recombination. However, lidocaine and articaine (Septanest®) were reportedly incapable of inducing chromosomal mutation or recombination.¹³ No genotoxicity was associated with the use of any of the drugs examined in this study. These results are in partial agreement with the literature, and differ only with the previous report of prilocaine genotoxicity.¹³

It has been demonstrated that the percentage of cells with polyploidy and endoreduplication increases upon exposure to prilocaine hydrochloride and procaine hydrochloride both in the presence and absence of exogenous metabolic activation.¹⁴ These results indicate that such chemical agents are potentially genotoxic to mammalian cells.¹⁴ However, this study did not show genotoxic action of prilocaine, possibly because it was used at the recommended dose per kilogram of weight.

Nuclear condensation and fragmentation of chromatin were previously observed in cells treated with prilocaine.¹⁵ DNA fragmentation was also induced by prilocaine

Table 1 Median and interquartile ranges of the frequency of micronuclei for each group ($n=80$).

Group	Median	Interquartile range
Lidocaine – 1 day	1.00a	3.00
Lidocaine – 5 days	0.50a,c	2.00
Mepivacaine – 1 day	1.00a	1.00
Mepivacaine – 5 days	1.00a	2.00
Articaine – 1 day	1.00a	2.00
Articaine – 5 days	0.00a,c	1.00
Prilocaine – 1 day	0.00a,c	1.00
Prilocaine – 5 days	0.00c	1.00
Negative control – 1 day	1.00a,c	2.00
Negative control – 5 days	0.00a,c	4.00
Cyclophosphamide ^a	10.00d	3.00

^a Positive control. Results with different letters differ significantly ($p < 0.05$).

treatment in a dose- and time-dependent manner, with maximal effects observed at a concentration of 5 mM after 12–48 h of exposure.¹⁵ Together with this study, these data show that using prilocaine at the recommended dose per kilogram of body weight cannot cause genetic damage.

Lidocaine and prilocaine are mainly metabolised in the liver and subsequently hydrolysed by amide esters, releasing the monocyclic aromatic amines, 2,6-dimethylaniline (DMA) and 2-methylalanine (MA), respectively.⁵ Other anaesthetics that contain a fraction of DMA include bupivacaine, mepivacaine and ropivacaine.⁵

The main carcinogenic mechanism of aromatic amines such as DMA and MA occurs when cytochrome P450 metabolises these compounds into derivatives of N-hidroxila.⁵ DMA and MA can be further metabolised by their conjugation to reactive metabolites. The DNA lesion has been described for DMA, but not for MA. The formation of DNA adducts is thought to be a possible mechanism by which these compounds exert some of their carcinogenic effects.¹⁶

However, the International Agency for Research on Cancer (IARC) reported no carcinogenic effects of DMA in humans, although there is sufficient evidence of its carcinogenicity in rats.⁵ This may explain the greater numbers of micronuclei observed in the lidocaine group (regardless of exposure time) compared to the prilocaine group and the significant difference between the groups exposed to lidocaine for 1 day and prilocaine for 5 days ($p=0.0466$). Although the frequency of micronuclei in the lidocaine-exposed group was not significantly different from that of the negative control group, the higher frequency of micronuclei in the lidocaine-exposed group (compared to the prilocaine group) may be associated with the effects of DMA, a metabolic product of lidocaine.

There are no literature reports regarding the genotoxic or mutagenic potential of mepivacaine. In this study, mepivacaine exhibited no genotoxicity, regardless of exposure time. However, exposure of mepivacaine for 1 and 5 days was significantly different from the group exposed to prilocaine for 5 days ($p<0.05$). Mepivacaine also contains a fraction of DMA,⁵ which may explain the higher number of micronuclei observed in this group, although the frequency of micronuclei was not significantly different from the negative control group.

Mutagenicity studies *in vitro* and *in vivo* revealed no genotoxic potential of articaine (CAS 23964-58-1) until the maximum tolerated dose was reached. In agreement with the data in this study, another articaine preparation (Septanest® SP; 4% articaine HCl and epinephrine 1:100,000)¹⁷ exhibited no genotoxic effects in an *in vivo* study using the recommended dose per kilogram of weight. Although articaine belongs to the amide group of local anaesthetics (which also includes lidocaine, prilocaine and mepivacaine), it is metabolised by cholinesterase in serum plasma into articainic acid *via* hydrolysis. Articainic acid is an inactive metabolite and is partially metabolised in the kidney into articainic acid glucuronide, rather than a potentially genotoxic aromatic amine.¹⁸ This result may partially explain the absence of genotoxicity upon articaine exposure. However, the 1-day articaine exposure group was significantly different from the 5-day prilocaine exposure group ($p=0.0364$). This may be due to the presence of an

outlier in the articaine group, which was well above the overall median frequency of micronuclei.

Conclusion

In the present study, there was no increased frequency of micronuclei upon exposure to any of the local anaesthetics tested (lidocaine, mepivacaine, articaine and prilocaine) when used at the recommended dose per kilogram of body weight, with either a single exposure or upon repetitive administration. However, other tests that assess genotoxicity and mutagenicity should be applied to definitively determine that the repetitive use of these anaesthetics has no genotoxic or mutagenic activity.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Moore PA, Hersh EV. Local anesthetics: pharmacology and toxicity. *Dent Clin North Am.* 2010;54:587–99.
2. Mariano RC, Santana SI, Coura GS. Análise comparativa do efeito anestésico da lidocaína 2% e da prilocaína 3%. *Revista Brasileira de Cirurgia e Implantodontia – BCI.* 2000;7:15–9.
3. Jastak JT, Yagiela JA. Vasoconstrictors and local anesthesia: a review and rationale for use. *J Am Dent Assoc.* 1983;107:623–30.
4. Smith MT. The mechanism of benzene-induced leukemia: a hypothesis and speculations on the causes of leukemia. *Environ Health Perspect.* 1996;104:1219–25.
5. Duan JD, Jeffrey AM, Williams GM. Assessment of the medicines lidocaine, prilocaine, and their metabolites, 2,6-dimethylaniline and 2-methylaniline, for DNA adduct formation in rat tissues. *Drug Metab Dispos J.* 2008;8:1470–5.
6. Flores M, Yamaguchi UM. Micronucleus test: an evaluation for genotoxic screening. *J Health Res.* 2008;1:337–40.
7. Fenech M, Holland N, Chang WP, et al. The HUMAN MicroNucleus Project – an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res.* 1999;428:271–83.
8. MacGregor JT, Heddle JA, Hite M, et al. Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutat Res.* 1987;189:103–12.
9. Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat Res.* 1992;271:69–77.
10. Evans HJ. Historical perspectives on the development of the *in vitro* micronucleus test: a personal view. *Mutat Res.* 1997;392:5–10.
11. Ramírez A, Saldanha PH. Análise crítica de grupos controle no teste de micronúcleo na mucosa bucal. *Genet Mol Biol.* 1998;21:140.
12. Perusse R, Goulet JP, Turcotte JY. Contraindications to vasoconstrictors in dentistry: part I. *Oral Surg Oral Med Oral Pathol.* 1992;74:679–86.
13. Schneider LE, do Amaral VS, Dähl RR, et al. Assessment of genotoxicity of lidocaine, prilonest and septanest in the drosophila Wing-Spot test. *Food Chem Toxicol.* 2009;47:205–8.
14. Hagiwara M, Watanabe E, Barrett JC, et al. Assessment of genotoxicity of 14 chemical agents used in dental practice: ability to induce chromosome aberrations in Syrian hamster embryo cells. *Mutat Res.* 2006;603:111–20.

15. Nakamura K, Kido H, Morimoto Y, et al. Prilocaine induces apoptosis in osteoblastic cells. *Can J Anaesth*. 1999;46:476–82.
16. Preston RJ, Williams GM. DNA-reactive carcinogens: mode of action and human cancer hazard. *Crit Rev Toxicol*. 2005;35:673–83.
17. Leuschner J, Leblanc D. Studies on the toxicological profile of the local anaesthetic articaine. *Arzneimittelforschung*. 1999;49:126–32.
18. Snoeck M. Articaine: a review of its use for local and regional anesthesia. *Local Reg Anesth*. 2012;5:23–33.